related to iPSC-derived T cell therapies. However, a major obstacle toward the use of T cells for the generation of iPSCs is that specialized cells such as T or B lymphocytes are not readily amenable to cellular reprogramming and often fail to dedifferentiate into bona fide pluripotent cells. To develop a robust non-integrating plasmid-based system for T cell reprograming, we applied both genomics and functional screening approaches to identify novel reprogramming factors that could markedly improve reprogramming of terminally differentiated somatic cells, including T cells. To enhance cellular reprogramming and induce naïve pluripotency, we used our previously described stage-specific media supplemented with small molecule drivers of naïve pluripotency and inhibitors of differentiation (MEKi, GSK3i, ROCKi and TGFbi). Using the new set of reprogramming factors that includes the master regulator of pluripotency gene *OCT4* and optimized medium formulations, iPSCs were generated in a highly efficient manner from T cells derived from multiple donors, with the fraction of TiPSCs reaching 7-15% of the population by day 18 post induction of cellular reprogramming and further increasing to 36-50% at day 26, enabling the ability for multiplexed engineering at the iPSC stage. To generate clonal TiPSC clones, we sorted single reprogrammed cells into 96-well plates and expanded multiple TiPSC clones for extensive characterization and selection. Quantitative PCR analysis revealed that all TiPSC clones were free of reprogramming plasmids and no longer had the potential to dedifferentiate. RNA sequencing data confirmed that the TiPSC clones were equivalent to control iPSC lines and pluripotency was further confirmed by the ability of the TiPSC clones to differentiate into representatives of the 3 germ layers in a trilineage differentiation assay. The generated feeder-free and single cell-derived TiPSCs showed enhanced expression of naïve pluripotency markers (e.g. KLF4, DMNT3L, PRDM14) and lower expression of primed pluripotency markers (e.g. THY1, OTX2, ZIC2). Importantly, TiPSC clones differentiated efficiently into homogenous T cell populations and maintained normal karyotype over extended culture duration. Next, we tested if the generated TiPSC clones maintained stable pluripotent profile and genomic integrity following stress-inducing manipulations including multiplexed engineering, subcloning and cryopreservation. Selected cryopreserved TiPSC clones were thawed and engineered by CRISPR-mediated insertion of CAR into the TCR alpha constant locus, re-cloned at the single cell level and re-banked. The engineered CAR+ TiPSC clones continued to maintain a homogenous pluripotent phenotype and normal karyotype, and differentiated into CAR T cells that exerted effective cytotoxicity against target cancer cells. Collectively, our data describe improvements in the generation of footprint-free and single cell-derived naive TiPSCs that are amenable for multiple rounds of multiplexed engineering, subcloning and cryopreservation and facilitate the replacement of donor-derived T cells as the preferred source for a more consistent, homogenous, cost effective and off-the-shelf CAR T cell product.

77. Precise Targeting of AML with First-inClass OR / NOT Logic-Gated Gene Circuits in CAR-NK Cells

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Background: Given the poor prognosis and long-term survival of relapsed/refractory acute myeloid leukemia (AML) patients, more efficacious therapies are greatly needed. While chimeric antigen receptor (CAR) cell therapies have provided some extraordinary clinical responses, developing effective CAR cell therapies for AML has been challenging due to: (a) the lack of a single target antigen robustly expressed across both AML leukemic stem cell (LSC) and immature leukemic blast cell subpopulations, and (b) the lack of truly AML-specific target antigens, since current targets are also expressed on healthy tissues and may result in off-tumor toxicity. Using logic-gated gene circuits, we are engineering CAR-NK cells to overcome these longstanding challenges. **Methods:** To maximize clearance of AML tumor cells and minimize toxicities, we used a proprietary bioinformatics pipeline to identify the optimal combinations of AML and healthy tissue target antigens to incorporate into OR and NOT logic-gated CAR gene circuits. These first-in-class CD33 OR FLT3 NOT Endomucin (EMCN) gene circuits enable allogeneic CAR-NK cells to target CD33 and/or FLT3 expressing AML tumor cells (LSCs and blasts) but not healthy FLT3+ hematopoietic stem cells (HSCs). **Results:** First, for the CD33 OR FLT3 activating CAR (aCAR) portion of the logic circuit, we demonstrated that engineered primary human NK cells expressing both CD33 and FLT3 aCARs exhibited up to 75% cytotoxicity and significant cytokine secretion (GrB, IFN-g, and TNF-a) against multiple leukemia cell lines in vitro, including MOLM13, THP1, and SEM. Importantly, these CD33 OR FLT3 CAR-NK cells also significantly reduced tumor burden and improved mouse survival within a MOLM13 xenograft leukemia model. Second, for the NOT gate portion of the logic circuit to protect FLT3+ healthy HSCs, we developed inhibitory CARs (iCARs) (NOT gates) that recognize the HSC-specific EMCN surface antigen, which is expressed on up to 70% of healthy HSCs but not AML cells. We demonstrated that FLT3 aCAR-NK cells engineered with an EMCN-specific iCAR protected over 50% of FLT3+ EMCN+ cells from FLT3 aCAR-mediated cytotoxicity. Next, to more closely replicate a clinical context, we mixed FLT3+ EMCN- (AML-like) and FLT3+ EMCN+ (HSC-like) target cells and demonstrated that FLT3 NOT EMCN CAR-NK cells exhibit preferential killing of FLT3+ EMCN- target cells, demonstrating that our NOT-logic gene circuit controls NK-mediated responses on a cell-by-cell basis. **Conclusion:** This work represents the first time NK cells have been engineered with OR and NOT logic-gated CAR gene circuits, wherein the OR gate provides increased AML

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LSC/blast tumor clearance (to prevent relapse), and the NOT gate protects healthy HSCs from offtumor toxicity, which may preserve hematopoiesis and mitigate the need for bone marrow transplant. Beyond AML, logic-gated CAR-NK cell technology has applicability to other cancer-associated antigens limited by potential off-tumor toxicity.

Gene Therapies for Hemoglobinopathies

78. Early Results from a Phase 1/2 Study of ARU-1801 Gene Therapy for Sickle Cell Disease (SCD): Safety and Efficacy of a Modified Gamma Globin Lentivirus Vector and Reduced Intensity Conditioning Transplant

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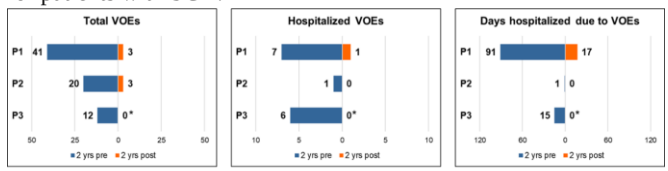
ARU-1801 is an investigational gene therapy drug product to treat sickle cell disease, consisting of autologous CD34+ hematopoietic stem cells and progenitors (HSPCs) transduced with a lentiviral vector (LV) encoding a modified γ-globin^{G16D} gene. It is being evaluated in patients with SCD in the ongoing Phase 1/2 MOMENTUM study (NCT02186418). Preliminary studies in SCD mice have suggested HbF^{G16D} may have a more potent anti-sickling effect than wild-type HbF. As a high potency anti-sickling globin, HbF^{G16D} is believed to allow ARU-1801 to be effective with reduced intensity conditioning (RIC), resulting in fewer toxicities and lower resource utilization than myeloablative approaches, expanding access to gene therapy to a broader group of SCD patients. Here, we present long-term clinical data on the first two patients (P1 and P2) and 10-month follow-up on patient 3 (P3), the first patient treated with a newer manufacturing process (Process II) to improve engraftment and increase HbF^{G16D} expression. As of December 2021, 3 patients treated with ARU-1801 have follow-up of >9 months. ARU-1801 demonstrated a favorable safety profile with no treatment-related adverse events to date. RIC resulted in neutrophil engraftment within 7-9 days (median 7 days), and platelet engraftment within 6-12 days (median 7 days). Under the initial manufacturing process, P1 has shown steady VCN of 0.2, stable expression of 20% HbF^{G16D} and 31% total anti-sickling globin (ASG, composed of endogenous HbF, HbA2 and ARU-1801-derived HbF^{G16D}), with 64% F-cells at 2 years post ARU-1801 infusion. P2 had a sub-therapeutic exposure to melphalan secondary to renal hyperfiltration and rapid clearance of melphalan, resulting in lower engraftment. Despite lower engraftment (VCN of 0.1) and hence lower HbF^{G16D} levels, P2 maintained stable 22% ASG expression and

36% F-cells at 2 years post ARU-1801 infusion due to sustained increases in endogenous HbF and HbA2. Under the new manufacturing process, P3 has demonstrated a stable VCN of 0.7 (latest measurement at month 9) with 41% ASG expression and 27% HbF^{G16D} at month 10. Furthermore, P3 has 92% F-reticulocytes at month 6, showing near pan-cellular expression of HbF. Treatment with ARU-1801 has resulted in remarkable improvement in clinical outcomes. As shown in Figure 1, patients had 12-41 VOEs (median, 21) in the 24 months prior to treatment with ARU-1801 and were hospitalized for 1-7 of those VOEs (median, 6). Through 24 months post ARU-1801 treatment, Patients 1 and 2 have seen 93% and 85% reductions in the number of VOEs, and Patient 3 has had no VOEs through 10 months of follow-up, a 100% reduction. The corresponding

cumulative days in hospital associated with those VOEs has decreased from 1-91 days (median, 15) to 0-17 (median, 0), representing an average 93.8% reduction. These results are an encouraging sign of the therapeutic benefit of ARU-1801 with RIC for patients with SCD.



79. Immunereconstitution in Transfusion Dependent Beta-Thalassemia Patients Treated with Hematopoietic Stem Cell Gene Therapy

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Transfusion-dependent β-thalassemia (TDT) is a disorder due to mutations in the gene encoding the ß-globin chain causing a reduced or absent production of hemoglobin A leading to severe anemia and lifelong transfusion dependence.Gene therapy has been recently accepted as a possible alternative to the only curative treatment

represented by allogeneic bone marrow (BM) transplantation. We developed a gene therapy approach based on autologous mobilized hematopoietic stem cell transduced by LV vector, expressing human ß-globin gene, administered by intrabone injection, following a myeloablative conditioning (NCT02453477). Nine patients with severe TDT with different genotypes have been treated with a drug product with a median cell dose of $19.5x10^6$ $CD34^+$ cells/kg, a transduction efficiency from 38 to 77% and a median vector copy number/genome (VCN) in bulk $CD34^+$ cells of 0.9 (range 0.7-1.5). Overall, gene therapy was generally well-tolerated with no adverse events related to the investigational product. No severe infectious-related adverse events were reported, except for those related to neutropenia as expected after conditioning. Insertion site analysis demonstrated highly polyclonal engraftment with no clonal dominance. Clinical outcome showed a reduction of transfusion requirement both in frequency and volume in adult patients up to more than 50%. Among the pediatric patients, 4 out of 6 discontinued transfusions shortly after gene therapy and are transfusion-independent at the last follow-up (up to 60 months). A robust and persistent engraftment was observed in 7 out of 9 patients, with a marking of BM progenitors that, in engrafted patients, ranged between 25.3 and 79.8% and with a median VCN in $CD34^+$ cells of 0.53 (range 0.34-2.21). As a relevant target, BM erythroid cells were stably marked (VCN range 0.3-2.5).Similar values were retrieved in the myeloid compartment and B lymphocytes while a lower VCN (range 0.12-1.48) was observed in $CD3^+$ cells. Focusing on lymphocytes, different rates of cell count increase were reported in B and T cells. In particular, $CD4^+$ T cells remained below the normal range for a prolonged period in all the patients, resulting in an inverted CD4/ CD8 ratio, consistent with reported allogeneic bone marrow transplant experience. Flow-cytometry, TREC and KREC evaluation performed at different time-points showing a slow but progressive recovery of immunocompetency. Importantly, despite a slow increase in the number, both innate and adaptive immune cells showed good response to mitogens and pathogens with an adequate antibody titer documented in response to vaccinations. All patients were enrolled in a long-term follow-up study that will provide results on long-term clinical efficacy and safety of this gene therapy.

80. Multiplex Base Editing of Hematopoietic Stem and Progenitor Cells to Enrich Therapeutic Cells Post Engraftment

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[1]Fred Hutchinson Cancer Research Center, Seattle, WA,[2]The Broad Institute of Harvard and MIT, Cambridge, MA,[3]Harvard University, Cambridge, MA,[4]St. Jude Children Research Hospital, Memphis, TN,[5]University of Washington, Seattle, WA A significant obstacle in current hematopoietic stem cell (HSC) gene therapy studies is the inability to consistently achieve sufficiently high engraftment of engineered cells to provide long-term therapeutic efficacy. Here we propose a strategy to increase engraftment of genome-edited cells after transplantation using multiplex base editing. Base editors are ideal for multiplexing since they introduce precise genetic alterations without double-stranded DNA breaks and thus prevent risk for chromosomal translocations. CD34+ cells were simultaneously edited both at a therapeutic gene and at a selection gene for evaluation in transplantation studies. As therapeutic target, we focused on the gamma-globin (HBG) locus for the reactivation of fetal hemoglobin (HbF) to treat hemoglobinopathies. This target has already been validated using CRISPR/Cas9-based approaches in several pre-clinical studies, including by our group. As edit that can be enriched by selection, we investigated the myeloid differentiation antigen CD33. Since CD33 is widely expressed on neoplastic myeloid cells, a wide array of CD33-directed drugs has been developed and tested. Previous findings demonstrated that inactivation of CD33 in HSCs by CRISPR/Cas9-editing does not impact the engraftment and multilineage differentiation potential of these cells. We used the adenine base editor ABE8e as editing platform, which was recently evolved from ABE7.10 for more robust activity. Human CD34+ cells electroporated with ABE8e mRNA targeting both the HBG and CD33 sites were transplanted in the murine model. Editing efficiency in infused cells averaged 40% for HBG and 80% for CD33. Notably, clonal assays demonstrated that about 75% of treated cells displayed edits at both targets in the same cell. Engraftment was comparable between the multiplex-edited group and the mock electroporated group. Furthermore, editing levels in peripheral blood (PB) remained high for both targets during the course of the experiment. Monocytes generated from engrafted cells displayed considerably reduced CD33 expression. Treatment of the animals with gemtuzumab ozogamicin confirmed resistance of edited cells to this highly potent CD33 antibody-drug conjugate. To further validate our multiplex editing strategy in an autologous transplantation setting, we utilized our nonhuman primate model described previously (Humbert et al., STM 2019). The CD34+CD90+ HSC-enriched cell population was edited with the same ABE8e-based approach as described above, yielding editing efficiencies in infused cells averaging 50% and 70% for HBG and CD33, respectively, with 70% containing edits at both targets. At 3 months post-transplantation, editing measured in total nucleated cells stabilized at about 10% (HBG) and 20% (CD33). CD33 expression measured in PB granulocyte was reduced to 65% as compared to >90% in a control animal, and HbF expression, as determined by F-cell frequency, reached 12% of total red blood cells. We will next use CD33-directed drugs in this animal to determine the in vivo ability to efficiently enrich for CD33- and thus HBG-edited cells as a means to enhance HbF to therapeutic levels for hemoglobinopathies. Together, our results demonstrate efficient engraftment and persistence of multiplex base edited CD34+ or CD34+CD90+ HSC-enriched cells in murine and NHP models. If enrichment is successful, this approach could serve as the basis for a broadly applicable method to treat other genetic blood diseases.

81. In Vivo HSC Gene Therapy for Hemoglobinopathies: A Proof of Concept Evaluation in Rhesus Macaques

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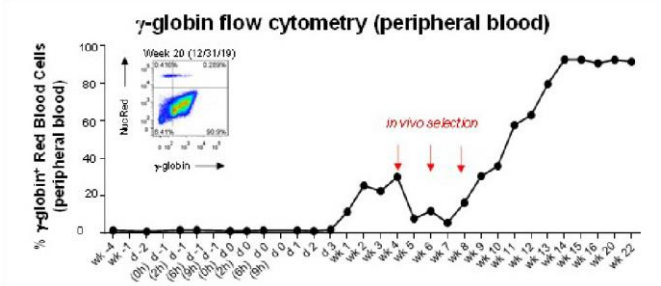
Current gene therapy or genome editing studies for hemoglobinopathies require highly sophisticated medical facilities to perform hematopoietic stem cell (HSC) collections/selections and genetic modifications. In addition, patients receive high-dose chemotherapy to facilitate engraftment of gene-modified cells. Thus, current gene therapy protocols will not be accessible to most patients suffering from hemoglobinopathies. Here we describe a highly portable and scalable approach using in vivo HSC gene therapy to potentially overcome these limitations. The central idea of our in vivo HSC gene therapy approach is to mobilize HSCs from the bone marrow, and while they circulate at high numbers in the periphery, transduce them with an intravenously injected HSC-tropic, helper-dependent adenovirus HDAd5/35++ gene transfer vector system. Transgene integration is

either achieved by a Sleeping Beauty transposase (SB100x) in a random pattern or by homology-directed-repair into a safe genomic harbor site. Currently, an in vivo selection system (involving the mgmt^{P140K} gene/low-dose O^{6}BG/BCNU) is employed to achieve >80% marking levels in peripheral blood cells. We demonstrated safety and efficacy of our approach in mouse models for thalassemia intermedia, Sickle Cell Disease, and hemophilia A, where we achieved a phenotypic correction. We now present data in 3 rhesus macaques. We show that treatment with G-CSF/AMD3100 resulted in efficient HSC mobilization into the blood circulation and subsequent intravenous injection of the HDAd5/35++ vector system (total 1-3 x10^{12} vp/kg, in two doses) was well tolerated. The longest follow-up thus far is 24 weeks after in vivo HSC transduction with a human-γ-globin expressing HDAd5/35++ vector. After in vivo selection with O^{6}BG plus low dose of BCNU, γ-globin marking in peripheral red blood cells rose to ~90% and was stable for the duration of the study (see Figure). γ-globin levels in red blood cells were ~18% of adult α1-globin (by HPLC). No abnormalities in genome and transcriptome analyses of animal #1 were found at the time of scheduled necropsy. We show that a new prophylaxis regimen (dexamethasone, IL-6R, IL-1bR antagonists, saline bolus IV) was able to mitigate all side effects associated with intravenous HDAd5/35++ vector administration. Analysis of day 3 bone marrow showed 30% transduced HSCs. Vector DNA biodistribution studies demonstrated very low or absent transduction of most tissues (including testes and CNS). Analysis of bone marrow showed efficient, preferential HSC transduction and re-homing of transduced CD34$^+$CD90$^+$ cells to the bone marrow. At week 4, about 5% of progenitor colony-forming cells demonstrated stable transduction with integrated vector, and this frequency increased after starting the in vivo selection. The level of human mgmt^{P140K} mRNA expression in PBMCs also increased after in vivo selection. This is the first proof-of-concept study that in vivo HSC gene therapy could be feasible in humans without the need of high-dose chemotherapy

conditioning and without the need for highly specialized medical facilities.



82. Hematopoietic Reconstitution and Lineage Commitment in HSC Gene Therapy Patients Are Influenced by the Disease Background

Andrea Calabria[1], Giulio Spinozzi[1], Fabrizio Benedicenti[1], Daniela Cesana[1], Luca Del Core[1,2], Serena Scala[1], Samantha Scaramuzza[1], Maria Rosa Lidonnici[1],
Alessandra Albertini[1], Simona Esposito[1], Fabiola De Mattia[1], Maryam Omrani[1], Valeria Calbi[1], Francesca Fumagalli[1], Marco Grzegorczyk[2], Ernst Wit[3], Giuliana Ferrari[1,4], Luigi Naldini[1,4], Alessandro Aiuti[1,4], Eugenio Montini[1]

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Lentiviral vector (LV) based hematopoietic stem cell (HSC) gene therapy (GT) applications have shown a favorable efficacy and safety profile for the treatment for a variety of genetic diseases. While this is a promising strategy for the treatment of diverse diseases, several factors, including transplantation protocols, patients' age and the underlying genetic disease, may impact on the kinetics of hematopoietic reconstitution, lineage specification, efficacy, and safety. To understand the impact of patient-specific factors and disease background on the hematopoiesis after transplantation, we studied the clonal reconstitution and multilineage potential over time in 48 HSC-GT patients affected by 3 different diseases: 29 with metachromatic leukodystrophy (MLD, a neurodegenerative lysosomal storage disorder), 10 with WiskottAldrich syndrome (WAS, a B and T cell immunodeficiency) and 9 with β-thalassemia (β-Thal, a hemoglobinopathy). As reported previously, these HSC-GT treatments resulted in clinical benefits for most patients. We analyzed the LV genomic Integration Sites (IS), a genetic marker for clonal identity, from the DNA of CD34$^+$ cells as well as myeloid, B, T and erythroid cell lineages purified from blood and bone marrow, harvested at different time points after HSC-GT (longest follow up to 9 years). Integration site analysis yielded >3 million unique IS and showed highly polyclonal reconstitution, multilineage marking and no signs of genotoxicity in all patients.Regardless of disease background, all patients showed a similar pattern of hematopoietic reconstitution over time,

characterized by: an early phase up to 9 months after GT, where the myeloid cells are highly polyclonal whilst T and B cells have a less complex repertoire; a second phase of 9 up to 18 months, where the polyclonality of lymphoid cells increases; and a third phase where the complexity of the lineages decreases and stabilizes. During the early phase of hematopoietic reconstitution an average of 80,000 active hematopoietic stem and progenitor cell (HSPCs)/patient contributed to hematopoiesis. However, the active HSPC pool decreased to an average 11,000 after 9 months, suggesting that short lived progenitors have a relevant role in sustaining the early phases of hematopoietic reconstitution. Differences across diseases were found when we analyzed the multilineage potential of individual clones and their commitment towards a specific lineage over time. In MLD patients multilineage clones reached a proportion of 75% on the total engineered clones, decreased over time to 50%, remaining stable thereafter. Myeloid-committed clones increased over time and stabilized at 40%. The decrease in multilineage clones over time was also observed in WAS and β-Thal patients. However, in WAS patients we did not observe a concomitant increase in myeloid committed cells rather an increase of the commitment in the T-cell lineage, as expected by the selective advantage in T cells, whereas in β-Thal patients several clones showed erythroid commitment.These novel data suggest that the disease condition influences the proportion and the type of lineagecommitted cells over long periods of time, and that the engrafted HSPC pool respond to the disease-specific physiopathology in a dynamic fashion to restore normal hematopoiesis.

83. Lentiviral Mediated Gene Therapy for Pyruvate Kinase Deficiency: Updated Results of a Global Phase 1 Study for Adult and Pediatric Patients

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Camino de Gaisse[1,2], Sol Sanchez[1,2], Bertil Glader[5,9], May Chien[5,9], Oscar Quintana-Bustamante[2,6,7], Brian C. Beard[10], Kenneth M. Law[10], Miriam Zeini[10], Grace Choi[10], Eileen Nicoletti[10], Gayatri R. Rao[10], Maria Grazia Roncarolo[3,4,5], Juan A. Bueren[2,6,7], Jonathan D. Schwartz[10], José C. Segovia[2,6,7]

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Introduction: Pyruvate kinase deficiency (PKD) is a rare inherited hemolytic anemia that results from mutations in the *PKLR* gene leading to decreased red cell pyruvate kinase activity and impaired erythrocyte metabolism. Manifestations include anemia, reticulocytosis, splenomegaly and iron overload, and may be lifethreatening in severely affected individuals. Current treatments are limited to blood transfusions, iron chelation therapy, and splenectomy which are associated with significant side effects. Preclinical studies in a clinically relevant PKD murine model have demonstrated that infusion of gene-modified Lin^- bone marrow (BM) cells may ameliorate the PKD phenotype. Based on compelling preclinical data, a global Phase 1 clinical trial RP-L301-0119 (NCT04105166) is underway to evaluate the feasibility and safety of lentiviral mediated gene therapy in adults and pediatric subjects with severe PKD. **Methods:** A total of 6 adult and pediatric subjects with severe PKD (defined as severe and/or transfusion-dependent anemia despite prior splenectomy) will be enrolled. Peripheral blood (PB) hematopoietic stem cells (HSCs) are collected on 2 consecutive days via apheresis after mobilization with granulocyte-colony stimulating factor (G-CSF) and plerixafor. $CD34^+$ HSCs are enriched, transduced with PGK-coRPKWPRE lentiviral vector, and cryopreserved. Following release testing of the investigational product (IP), RP-L301, myeloablative conditioning with therapeutic drug monitoring guided busulfan is administered over 4 days. RP-L301 is then thawed and infused. Patients are followed for safety and efficacy assessments for 2 years post-infusion. **Results:** As of January 2021, 2 adult subjects have been treated. Patient 1 received $3.9x10^6$ $CD34^+$ cells/kg with mean vector copy number (VCN) of 2.73. At 3 months post-infusion hemoglobin has normalized, with PB VCN of 1.55. Patient 2 received $2.4x10^6$ $CD34^+$ cells/kg with mean VCN of 2.08. Neutrophil engraftment occurred within 2 weeks for both patients. No adverse events have been attributed to RP-L301. 6- and 3-month post-treatment data will be presented for Patients 1 and 2, respectively. **Conclusions:** Hematopoietic stem cell mobilization using G-CSF and plerixafor appears feasible and effective in adult PKD patients. RP-L301 was successfully manufactured to meet the required specifications for the Phase 1 clinical study and administered without short-term infusion related complications. Preliminary efficacy was evident in Patient 1 during the initial 3 months post-RP-L301 as demonstrated by hemoglobin normalization and PB genetic markings.

84. Base Editing of the -200 Region of the γ-globin Promoters Leads to Fetal Hb Reactivation and Rescues the Sickle Cell Disease Phenotype in Primary Patient Cells

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β-hemoglobinopathies, β-thalassemia and sickle cell disease (SCD), are caused by mutations affecting the production of the adult hemoglobin (Hb). Transplantation of autologous, genetically modified hematopoietic stem/progenitor cells (HSPCs) is an

attractive therapeutic option. The clinical severity of β-hemoglobinopathies is alleviated by the co-inheritance of mutations causing hereditary persistence of fetal Hb (HPFH) in adult life. To reactivate fetal γ-globin expression, nuclease-based editing approaches have been explored. Site-specific nucleases, however, generate double-strand breaks (DSBs) in the genome, raising safety concerns for clinical applications. Base editing (BE) allows the introduction of point mutations (C>T by cytidine base editors, CBEs; A>G by adenine base editors or ABEs) without generating DSBs.HPFH mutations in the promoters of the 2 γ-globin (*HBG1/2*) genes either disrupt the binding sites (BS) of fetal Hb (HbF) repressors or generate BS for HbF activators. In particular, mutations clustering ~200 nucleotides upstream of the *HBG* TSSs either reduce LRF binding (e.g. -197 C>T) or recruit KLF1 (-198 T>C). In this study, we used base editors to recapitulate HPFH mutations in the -200 region in the *HBG* promoters.First, we explored the BE system to introduce C>T mutations in the LRF BS. The absence of the canonical *Spy*Cas9 NGG PAM close to the LRF BS prompted us to test a variety of non-NGG CBEs including CBE-NRCH, CBE-SpG, CBE-SpRY and a novel BE containing NAA PAM Cas9 variant. CBEs edited 7 out of 8

cytidines of the LRF BS in an erythroid cell line (K562) with efficiencies of up to ~60%. These C>T conversions include not only known HPFH mutations but also HPFH-like mutations that can further impair LRF binding.We tested this strategy in HSPCs from SCD patients and achieved BE efficiencies of up to ~45%. A progenitor assay indicated no alteration in the multilineage differentiation of edited HSPCs. HSPCs were differentiated in mature red blood cells (RBCs). The expression of erythroid markers was similar in control and edited samples and the production of mature RBCs was not affected by the BE treatment. We observed a potent γ-globin reactivation with a high frequency of HbF^+ cells and a concomitant decrease in the HbS content/cell, as detected by RT-qPCR, HPLC and flow cytometry. Importantly, the pathological RBC sickling phenotype was substantially improved in the edited samples. Finally, we used GUIDE-seq coupled with targeted sequencing to evaluate the potential off-target activity of gRNAs disrupting the LRF BS.We then compared BE strategies targeting the -200 region of the *HBG* promoters that either disrupt the LRF BS or create a *de novo* KLF1 BS in SCD HSPCs. The superior efficiency in generating the KLF1 BS by ABEs was associated with higher levels of HbF in mature RBCs and colonies derived from erythroid progenitors, compared to a strategy merely disrupting the LRF BS. Similarly, the RBC sickling phenotype was further improved in the edited samples carrying the KLF1 BS.In conclusion, we developed efficient BE strategies to disrupt repressor BS or create activator BS in the *HBG* promoters that led to therapeutically relevant HbF levels. Validation of the above-described results in HSPCs *in vivo* will provide sufficient proof of efficacy and safety to enable the clinical development of base-edited HSPCs for the therapy of β-hemoglobinopathies.

Immune Responses to AAV Vectors

85. Declining FVIII Activity Following Hepatic AAV Gene Transfer Because of Translational Shutdown Linked to an Immune Response

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Adeno-associated virus (AAV) gene therapy aims to provide sustained replacement in diseases of protein deficiency, such as clotting factor VIII (FVIII) for the X-linked bleeding disorder hemophilia A (HA). Excitingly, liver-directed AAV-FVIII gene transfer led to complete correction of HA in clinical trials. However, these high levels of therapeutic expression were unstable and declined in years 2-4. We identified a sub-strain of BALB/c-HA mice that reliably forms an adaptive immune response against FVIII upon hepatic gene transfer with AAV8 vector expressing codon-optimized human FVIII under a liver-specific promoter. Administering mTOR inhibitor rapamycin for the eight weeks following gene transfer prevented formation of antibodies to FVIII and capsid, allowing vector readministration. Elimination of antibody formation resulted in initial FVIII activity

levels of 20-60% of normal while the mice were on immune suppression. However, in multiple experiments, average FVIII levels consistently declined to less than 5% by week 16 (with 50-100% of animals having undetectable levels, depending on the experiment). Therefore, this model allowed us to uncouple loss of FVIII expression from humoral immune responses. To counter potential cellular immune responses, we included monoclonal antibodies (mAb) in the rapamycin regimen. Combining rapamycin with a mAb to IL-15, a cytokine critical for NK and memory CD8 T cells, modestly preserved FVIII activity. We confirmed the effectiveness of blocking IL-15 signaling, and in fact improved upon this approach, by combining rapamycin with a Fc-silent mAb to the shared IL-2/IL-15 receptor beta chain (CD122). Eight weeks after immune modulation was stopped, livers of mice given anti-CD122 were still NK-depleted. In addition, Kupffer cells had lower expression of inflammatory molecules, suggesting that the improvement in FVIII activity levels by IL-15 blockade was due to suppression of an inflammatory immune response. Furthermore, we found CD8 T cell depletion with anti-CD8 after rapamycin treatment reduced the number of mice that entirely lost FVIII activity similarly to anti-IL-15 and anti-CD122 treated cohorts, indicating a contribution of CD8 T cells. However, the remaining FVIII activity was less than 5%. Together, the results suggest that multiple cell types contribute to the immune response, including CD8 T and NK cells. IHC stains revealed that rapamycin-treated animals that lost FVIII activity had no FVIII protein expression in the liver, while

those that had circulating levels due to IL-15 blockade showed FVIII expression in hepatocytes. Among animals that received immune suppression, mRNA levels were similar regardless of FVIII protein expression, and CD8 T cell depletion only modestly increased vector copy numbers compared to other rapamycin-treated animals. Interestingly, all transduced mice retained AAV copies that positively correlated with hFVIII mRNA levels but not FVIII protein expression. Mice that received vector without immune suppression—thus formed antibodies against FVIII—had 2-3 times higher gene copy numbers and mRNA levels but showed only few FVIII expressing hepatocytes. Therefore, expression was lost primarily because of a translational shutdown that could in part be prevented by dampening cellular immune responses. We are now investigating whether an unfolded protein response to FVIII contributed to the shutdown and the inflammatory response. A model of gradual loss of FVIII expression largely due to translational shutdown with a lesser contribution of hepatocellular injury/cytotoxicity would explain the observations in clinical AAV-FVIII gene therapy.

86. Requirements for Cross-Presenting Dendritic Cells and CpG Motifs in CD8$^+$ T Cell Response to AAV Gene Transfer

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viral DNA, trigger innate immune receptors and induce AAV capsid specific CD8$^+$ T cells. TLR9 sensing of CpG motifs in the AAV vector genome has been implicated in the literature to serve as a signal for activation of CD8$^+$ T cells. For instance, TLR9 signaling in plasmacytoid dendritic cells (DCs) leads to IFN I production, which in turn licenses conventional DCs to prime anti-capsid CD8$^+$ T cells. XCR1$^+$ DCs are most efficient in cross-presenting antigen to CD8$^+$ T cells and are comprised of organ-resident CD8α$^+$ DCs and migratory CD103$^+$ DCs. We quantified the capsid-specific CD8$^+$ T cell response by tetramer stain upon intramuscular (IM) injection of AAV2 vector (1x10^{11} vg/ mouse) containing an ovalbumin-derived surrogate epitope in C57BL/6 mice. To determine the putative role of these DCs, we used XCR1-DTR mice to deplete XCR1$^+$ DC by administration of diphtheria toxin (DT). Alternatively, we treated WT C57BL/6 mice with neutralizing antiCD103. C57BL/6 mice treated with anti-CD103 showed a significantly lower frequency of capsid-specific CD8$^+$ T cell response compared with controls. Depletion of XCR1$^+$ DCs gave a more dramatic outcome and resulted in nearly complete elimination of the response. Next, we evaluated the role of CpG motifs in the immune response. For that, a vector was constructed based on an entirely CpG-free expression cassette. It contained a CpG-free edited sequence of the coding region for human coagulation factor IX (FIX) and a synthetic intron under transcriptional control of a CMV enhancer/EF1α promoter combination and SV40 polyA signal. This cassette was inserted in between AAV2 ITRs and packaged into the AAV2- SIINFEKL capsid. C57BL/6 mice received IM injections (1x10^{11} vg/mouse) of AAV2SIINFEKL depleted of CpG motifs (AAV2-SIINFEKL-CpG$^-$) or CpG rich control vector (AAV2-SIINFEKL-CpG$^+$; containing native, not CpG depleted sequences). AAV-SIINFEKL-CpG$^-$ administration markedly reduced anti-capsid CD8$^+$ T cell response as observed by the decrease of % tetramer$^+$ CD8$^+$ T cells. In contrast, we did not observe differences in capsid-specific antibody formation between groups. Next, we evaluated the immune response to FIX in male hemophilia B (C3H/ HeJ F9$^{-/Y}$) mice injected IM with AAV1-CpG$^+$ or AAV1-CpG$^-$ vector (1x10^{11} vg/mouse, n=5/group). AAV1-CpG$^-$ induced a substantially reduced (~8 fold), but not completely absent, CD8$^+$ T cell infiltration and ~2 fold higher numbers of hFIX expressing muscle fibers, as quantitated by image analysis of immunofluorescent stained sections of skeletal muscle. We conclude that XCR1$^+$ DCs are the critical subset of conventional DCs that presents AAV capsid antigen to CD8$^+$ T cells, which in part occurs through XCR1$^+$CD103$^+$ DCs. Furthermore, CpG motifs are a major activation signal for CD8$^+$ T cell responses against capsid and transgene product in AAV muscle gene transfer but are not a major factor for antibody formation.

87. Defining and Overcoming Preexisting T-Cell Adaptive Immunity to SaCas9 CRISPRCas Genome Editors

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In vivo CRISPR-Cas genome editing has the potential to transform human medicine by directly correcting disease-causing mutations in affected cells in the body. Cas9 from *Staphylococcus aureus* (*S. aureus;* SaCas9) was the first orthologue discovered with high activity in mammalian cells that is small enough to be encoded in an AAV vector and has shown great clinical potential in preliminary *in vivo* studies.

One major concern regarding the durability of promising *in vivo* genome editing therapeutic strategies using Cas9 is the potential for immune rejection of Cas9-expressing edited cells. Pre-existing adaptive T cell immunity to the Cas9 variants derived from *Streptococcus Pyogenes* (SpCas9) and S.*aureus*, common human pathogen, have been reported. While SaCas9-specific T cells were identified, the adaptive T cell immune response to SaCas9 has not been fully characterized. Here we screened peripheral blood mononuclear cells (PBMCs) from healthy HLA-typed donors using an IFN-γbased enzyme-linked immune absorbent spot (ELISPOT) assay using a peptide library consisting of 15 amino acid long peptides with an 11 amino acid overlap that covered the entire coding region of SaCas9 to sensitively measure the SaCas9specific T cell response. We found that 24% (8 of 33) of donors screened exhibited

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Gene therapy using adeno-associated virus (AAV) has emerged as one of the most promising treatments for various genetic diseases.

However, the development of immune response to the viral vector or the transgene product might compromise the outcome for long-term success. Immune stimulatory CpG motifs, which are unmethylated in

a positive T cell response to SaCas9 that was CD4-restricted in 2 of 2 analyzed positive donors. Next, we performed an unbiased epitope screen to map immunodominant epitopes using our SaCas9 peptide library for two donors. For one donor, we defined the minimal 13 amino acid epitope that was recognized by SaCas9-specific CD4 T cells. Next, we performed alanine scanning of the minimal epitope and identified 7 positions that significantly reduced T cell recognition. We engineered corresponding alanine mutations into SaCas9 to determine whether these SaCas9 variants would retain high genome editing activity. Six of 7 SaCas9 alanine variants-maintained genome editing activity at levels comparable to wild-type SaCas9. In conclusion, our results illuminate the prevalence and type of T cell-mediated immunity towards SaCas9 and offer a proof-of-principle solution of engineering immune silent variants to overcome the challenge of preexisting adaptive T cell immunity to CRISPR-Cas genome editors.

88. Clinical Outcomes in Patients with and without Pre-Existing Neutralizing Antibodies to the Vector: 6 Month Data from the Phase 3 HOPE-B Gene Therapy Trial of Etranacogene Dezaparvovec

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Introduction: Etranacogene dezaparvovec is an investigational gene therapy for hemophilia B (HB) comprising an adeno-associated virus serotype 5 (AAV5) vector containing a codon-optimized Padua variant human factor IX (FIX) gene with a liver specific promoter. Although most gene therapy clinical studies exclude participants (pts) with pre-existing neutralizing antibodies (NAb) to the capsid serotype, early clinical studies and nonhuman primate data suggest that generally prevalent titers of anti-AAV5 NAbs may not preclude successful transduction with etranacogene dezaparvovec. **Aims and methods:** A Phase 3, Health Outcomes with Padua gene; Evaluation in Hemophilia B (HOPE-B; NCT03569891) was established to further assess efficacy and safety of etranacogene dezaparvovec in adults with HB. Adult males with severe or moderate-severe HB (FIX≤2%) on prior routine FIX prophylaxis were enrolled into this open-label, single-dose, single-arm, multinational trial. Pre-existing NAbs to AAV5 were assessed but not exclusionary. Participants entered a ≥6 month lead-in period, then received a single dose of etranacogene dezaparvovec ($2x10^{13}$gc/kg) without prophylactic immunosuppression. The co-primary endpoints are change in FIX activity at 26 and 52wks and 52wk annualized bleeding rate compared to lead in. Here, outcomes at 26 weeks in participants with and without pre-existing NAbs to AAV5 are analyzed using descriptive statistics and a correlation analysis. **Results:** 54 participants were dosed and completed 26 wks of follow-up, 31 (57.4%) had no AAV5 NAbs. Of the 23 (42.6%) with AAV5 Nabs at baseline (BL), the median titer was 56.9 (1^{st}-3^{rd} quartile 23.3-282.5) with a distribution representative of the general population. The max NAb titer was 3212. One participant with a NAb titer of 198 received a partial dose and was excluded from the assessment of NAb impact on efficacy. A single participant with a NAb titer of 3212 did not respond and remained on prophylaxis. All other participants (n=52) discontinued prophylaxis and remain prophylaxis-free at 26 weeks. No correlation of pre-existing NAbs with FIX activity was observed up to a titer of 678 (n=52, r=-0.28 [95% CI -0.51, 0.00], R^2=0.078). Mean FIX activity at 26 weeks was 32.7 IU/dl (min <2, max 90.4, 1^{st}-3^{rd} quartile 16.3-42.6, n=22) in participants with NAbs versus 41.3 IU/dl (min 8.4, max 97.1, 1^{st}-3^{rd} quartile 31.3-52.7, n=31) in those without. Most common treatment-related AEs were transient transaminitis requiring corticosteroids (2/23 pts with NAbs; 7/31 pts without), infusion-related reactions (5/23 pts with NAbs; 2/31 pts without), headache (2/23 pts with NAbs; 5/31 pts without) and influenza-like illness (4/23 pts with NAbs; 3/31 pts without). No deaths and no inhibitors to FIX were reported. **Conclusions:** FIX activity was similar in participants without and with pre-existing NAbs to AAV5 up to a titer of 678; there were insufficient data to assess a relationship with higher titer NAbs. No relationship between AAV5 NAbs and safety was observed. This study demonstrates for the first time successful treatment of patients with pre-existing NABs at generally prevalent levels with an AAV5 construct, supporting broad eligibility for AAV5-based therapies.

89. Abstract Withdrawn

90. IL-1a and IL-1b Are Essential for Inflammasome Independent CD8$^+$ T Cell Responses to Hepatic AAV Gene Transfer

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We previously demonstrated that liver directed gene therapy with low dose adeno-associated virus serotype 8 (AAV8) vector elicited a CD8$^+$ T cell response to the transgene product, resulting in the loss of expression in murine liver. Further, we showed that these CD8$^+$ T cell response were independent of TLR9 sensing, instead relying on the IL-1R1/MyD88 pathway and CD4$^+$ T cell help. Using proliferation of adoptively transferred, CellTrace violet labeled transgene specific CD8$^+$ T cells as a readout, we demonstrated that cross presenting

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$CD11c^+XCR1^+$ DCs are critical for MHC I antigen presentation. In the present study we sought to delineate the role of IL-1α, IL-1β and the role of inflammasomes in mediating cellular responses to the AAV encoded transgene. Further, we studied kinetics of $CD4^+$ and $CD8^+$ T cell activation following AAV mediated hepatic gene delivery. Since IL-1R1 is a cognate receptor for both IL-1α and IL-1β, we used antiIL-1α and anti-IL-1β to block IL-1 signaling. Wildtype (WT) C57BL/6 mice (n=8/group) were treated with anti-IL-1α, anti-IL-1β or both antibodies one day prior to intravenous (IV) injection with AAV8OVA ($1x10^9$ vg/mouse). Treatment was continued 2x/week for four weeks. Control WT-C57BL/6 mice (n=8) received only AAV8-OVA. PBMC were screened for OVA-specific $CD8^+$ T cells using class I MHC tetramer after 4 weeks. In order to study the role of inflammasomes, knockouts mice of specific inflammasomes (NLRP1, NLRP3, AIM-2 and Caspase-1) on C57BL/6 background (n=8) were injected with AAV8-OVA and OVA-specific $CD8^+$ T cells were quantified. To understand the activation kinetics of $CD4^+$ and $CD8^+$ T cells, AAV8OVA injected C57BL/6-WT mice were euthanized on days 3, 7, 10 and 14 (n=5). $CD4^+$ and $CD8^+$ T cells from liver, hepatic lymph nodes and spleen were further analyzed for expression of activation markers (CD44, CD69 and CD107a). Naive C57BL/6-WT mice (n=5) were used to obtain baseline status of $CD4^+$ and $CD8^+$ T cells. Consistent with earlier findings, 75% of control WT mice developed a $CD8^+$ T cell response to OVA. Blocking of either IL-1α or IL-1β reduced the number of mice (33% and 50% respectively) that developed a $CD8^+$ T cell response. Moreover, only 18% of mice developed a $CD8^+$ T cell response when both IL-1α and IL-1β were blocked. These results indicate that both IL-1α and IL-1β play a critical and in part redundant role in mediating $CD8^+$ T cell response to an AAV encoded transgene during hepatic gene delivery. Since inflammasomes are an important mediator of IL-1β driven immune responses, we tested if any of the inflammasomes were required. Similar to WT mice, knockout mice for different inflammasome developed OVA specific $CD8^+$ T cell response indicating that these cellular responses were independent of inflammasome machinery. Following AAV8-OVA administration, increased expression of CD44, CD69 and CD107a was observed on $CD4^+$ and $CD8^+$ T cells isolated from liver as compared to hepatic lymph nodes or spleen. Activated $CD4^+$ and $CD8^+$ T cells were observed in the liver as early as day 3 post AAV administration. In summary our results suggest that during AAV mediated liver gene transfer, both IL-1α and IL-1β mediates $CD8^+$ T cell activation to AAV encoded transgene, which is independent of any inflammasome machinery. Further, our results imply that these cellular responses are initiated in the liver.

91. Novel miRNA-Binding Sites That Recruit miR-652 and miR-223 in AAV Vector Designs Boost Transgene Levels and Synergistically Suppress Cell-Mediated Immunity

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Recombinant adeno-associated virus (AAV) vector gene therapy offers tremendous promise for the treatment for a variety of genetic diseases. Regulatory approvals for the treatment of two genetic diseases have already been received and clinical development for many more are on the horizon. Exciting advancements have been made in the gene delivery technologies, from the identification of novel AAV serotypes, to the development of novel vector delivery techniques. One of the challenges for long-term success of gene therapy is the development of immune response to the transgene product. This effect is attributed to the undesirable transduction of antigen presenting cells (APCs), which in turn triggers host immunity towards rAAV-expressed transgene products. miRNA-mediated regulation to detarget transgene expression from APCs has shown promise for reducing immunogenicity. Skeletal muscle has been considered a viable target for AAV vector-mediated gene transfer to achieve sustained production of secreted therapeutic proteins. We have previously shown that miR-142 mediated detargeting allows continued expression of transgene in myofibers, represses cytotoxic T cell response and blunts the activation of co-stimulatory signals. However, the combinatorial effect of more than one miRNA binding site in the 3'-UTR of the transgene on anti-transgene immunity has not been reported previously. In this study, we performed *in vitro* screening of binding sites for 26 miRNAs that were selected based on their high expression levels in APCs, such as dendritic cells (DCs) and macrophage cell lines, but low in myoblasts. We identified two novel miRNA binding sites, miR-652BS and miR-223BS, that are efficient at APC detargeting *in vitro*, either individually or in combination with miR-142BS. Intramuscular administration of rAAV1 vectors containing either miR-142+652BS or miR-142+223BS demonstrate higher transgene expression in skeletal myoblasts as compared to previously published detargeting constructs, with negligible antiOVA IgG production. Immunophenotyping of cells isolated from liver, spleen and muscle tissues revealed suppression of DC and costimulatory signals, and macrophage activation. In addition, there was a marked reduction in OVA specific CD8+ T cell response in those tissues accompanied by a reduction in the production of inflammatory cytokines, TNFα and IFNγ. Moreover, we present evidence that miR142-, miR-652-, and miR-223-mediated detargeting also leads to significant repression of Th17 response *in vivo*. Transgene detargeting mediated by the combination of miR-142BS and miR-652BS within the same vector cassette proves to be the most efficient at muting transgene specific immunity. Our approach, thus, advances the efficiency of miRNA-mediated detargeting to achieving synergistic reduction of transgene-specific immune responses and the development of safer and more efficient delivery vehicles for gene therapy.

Novel Factors in AAV Transduction and AAV Genomes

92. Chemical Mediated Recruitment of Epigenetic Modifiers Regulate AdenoAssociated Virus Episomal Transgene Expression

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Optimistic clinical data using AAV gene therapy has been observed for disorders of the muscle, blood, brain, and those affecting vision. In all these applications, the AAV vectors administered to humans have one thing in common: they are uncontrollable at the level of transgene expression. Following transduction, AAV vector genomes form circular concatemers and limited studies have demonstrated these episomes associate with histones, as well as transcriptional activators and repressors. These observations suggest a formal possibility that AAV episomes are, in part, restricted for transgene expression, alluding to the ability to modulate their epigenetic composition to enhance and/or repress the transcriptional activity. Based on these observations, it was hypothesized that AAV transgene expression can be controlled at the episomal level through targeted recruitment of epigenetic regulators of gene expression. We have developed a technology, known as chemical epigenetic modifiers (CEMs), that utilizes chemically induced proximity and endogenous epigenetic proteins to regulate specific chromosomal gene expression. The CEM technology was adapted to AAV vectors (termed AAV-CEMtrol) to investigate transgenic episome regulation post-transduction. Following AAV2 or AAV8-CEMtrol transduction in vitro, the exogenous addition of different CEMs that recruit distinct transcriptional activators (CBP/p300 or BRD4) to the recombinant AAV episome demonstrated dose-dependent and highly specific rAAV-borne reporter induction. These novel observations in well controlled and rigorous experiments demonstrate unambiguously, that AAV episomes are naturally restricted for expression in human cells and allude to the ability to enhance and potentially repress therapeutic transgene expression at a fixed AAV vector dose following in vivo administration. Towards that end, pharmacokinetic studies following a single intraperitoneal injection demonstrated the safety and bioavailability of the leading CEM in multiple tissues. Future experiments include mechanistic ChIP analyses to elucidate the exploited biology as well as performance investigations of AAVCEMtrol regulation in vivo in response to CEM administration. These data demonstrate the ability to significantly induce AAV transgene expression using particular CEMs that can recruit epigenetic regulatory

machinery to transgenic episomes. This novel approach may provide a novel approach to activate and potential repress AAV transgene expression towards controllable, and thus safer, AAV gene therapeutics.

93. The Human Silencing Hub (HUSH Complex) Is a Potent Regulator of AAV Transgene Silencing

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Therapeutic transgenes delivered by recombinant AAV (rAAV) vectors are known to be subject to transgene silencing as observed in several clinical trials. This poses a challenge towards achieving efficient gene expression, often requiring high vector doses. However, the latter has shown the potential to cause dose-dependent liver toxicity amongst other adverse effects. Importantly, the underlying silencing mechanisms impacting rAAV transgene expression are not well understood. In order to identify potential cellular pathways involved in rAAV transgene silencing/expression, we screened a panel of host factors known to be involved in epigenetic regulation of foreign DNA. This targeted screen revealed novel restriction factors of rAAV transduction, particularly components of the HUSH (HUman Silencing Hub) complex. The HUSH complex is known to be associated with H3K9me3-dense genomic regions and functions to repress cellular genes. The HUSH complex has also been shown to transcriptionally silence unintegrated retroviral genomes by recruiting NP220 to the viral DNA followed by methyltransferases and deacetylases. Here, we show here that CRISPR KO of individual HUSH complex members such as MPP8, PPHLN1, TASOR, SETDB1 and NP220 leads to a robust increase in AAV mediated transduction, both at the level of mRNA transcripts and translated protein. This observation was independent of AAV capsid serotype, promoter elements and observed in case of both single stranded and self-complementary rAAV vectors. Interestingly, rAAV transduction leads to increased expression of HUSH complex members like TASOR and PPHLN1, implying a role for host sensing mechanisms. Interaction of NP220 with the rAAV genome and how this changes the epigenetic landscape is currently under investigation. Lastly, we show that pharmacological inhibition of deactylases enhances rAAV transgene expression. Our approach underscores the importance of understanding host cell mechanisms that can silence rAAV transgenes. Moreover, these findings provide a roadmap towards exploring druggable aspects of rAAV genome expression and engineering the rAAV genome to potentially circumvent silencing.

94. Effects of Sexual Dimorphism and Genetic Background on AAV Tissue Transduction in Mice Following Intravenous Administration of a Diverse Capsid Pool

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